STUDIES ON THE HEXAPOD NERVOUS SYSTEM. IV. A CYTOLOGICAL AND CYTOCHEMICAL STUDY OF NEURONS AND THEIR INCLUSIONS IN THE BRAIN OF A COCKROACH, PERIPLANETA AMERICANA (L.)

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Inclusions commonly regarded as products of secretory activity have been described in the nerve cells of numerous and diverse insects (see reviews by Scharrer and Scharrer, 1954; Gabe, 1954; Van der Kloot, 1960). Much of this information has been obtained by employing selective, but poorly understood, staining reactions such as the aldehyde-fuchsin and chrome alum-hematoxylin techniques. Few attempts have been made to compare inclusions in different nerve cells of a single insect species using a variety of the more rigorous cytochemical staining procedures currently available. It might be expected that such a study would provide data germane to a problem which perennially faces the insect neurocytologist and neuroendocrinologist, namely, that of neuron classification. The present study is also considered basic to investigations of the fine structure of the cockroach nervous system now in progress at this laboratory.

Methods

By dissecting away the cranial and cervical integuments of live *Periplaneta* adults and late instar nymphs, the brain and retrocerebral complex were exposed. After severing the circumesophageal connectives, major nerves, and foregut distal to the corpora allata, the foregut was removed and carried to the appropriate fixative together with the brain and its associated retrocerebral complex. With practice this operation can be completed within two or three minutes, and certainly before any appreciable drying of the exposed organs occurs. A comparison with results obtained after exposing the brain and fixing it *in situ*, or after bathing the brain in Yeager's cockroach saline during the dissection did not justify modifying this procedure. Indeed, it was considered less damaging to remove the brain prior to fixation than after.

The choice of fixative was dictated by both the cytochemical staining technique intended, and the quality of cytological preservation required. Alcoholic and aqueous Bouin's, Lillie's acetic-alcohol-formalin (AAF; Lillie, 1954), 10% neutral formalin, and Helly's, were among those used. Preservation of neuronal inclusions in paraffin sections was best after Helly's. Following fixation, each specimen was dehydrated in half-hour changes of 70%, 95%, and absolute ethyl alcohol, cleared in methyl benzoate, washed briefly in benzene, and vacuum infiltrated with Fisher's Tissuemat (60–62° C.). Serial sections were cut at 5–7 μ. Adjacent sections were commonly mounted on alternate slides, thus allowing the cytological and cytochemical staining characteristics of the same inclusions to be compared.

Cytological features were elucidated by staining with Gomori's aldehyde-fuchsin (AF) as modified by Halmi (1952), or Bargmann's modification of Gomori's chrome alum-hematoxylin-phloxine (CAHP; Pearse, 1960). Additional information was obtained by staining Helly-fixed sections with the periodic acid-Schiff (PAS) method and counterstaining with sudan black B. While observing a particular region with a water-immersed objective (N.A. 1.25) several drops of acetone can be applied to remove the sudan black. This leaves the PAS-positive components. In this way the staining of inclusions by the two methods within a single cell was readily compared.

The cytochemical procedures used included the PAS reaction for polysaccharides after diastase digestion (Pearse, 1960), after aniline-acetic acid blocking (Lillie and Glenner, 1957), and after omitting periodic acid oxidation. In an attempt to decide whether lipids were responsible for PAS-positive reactions, freshly dissected brains were extracted with equal proportions of chloroform:methanol in a closed container at 60° C. for 24 hours. The PAS reaction was applied to gelatin-imbedded frozen sections of such extracted brains, as well as to frozen sections of brains which had been fixed in aqueous Bouin's first, and subsequently extracted with 60° C. pyridine. Frozen sections of aqueous Bouin-fixed brains served as controls.

Lipids were stained with sudan black B (SBB) in Helly-fixed paraffin sections, as well as in frozen sections of material which had been fixed either in aqueous Bouin's, or in neutral 10% formalin followed by postchromation (Baker, 1946). Controls consisted of aqueous Bouin-fixed, or unfixed brains, which had been extracted 24 hours either with 60° C. pyridine in a closed container, or with boiling chloroform:methanol in a reflux condenser. Baker's acid hematein (AH) test for phospholipids (Baker, 1946) was applied to gelatin-imbedded frozen sections. Helly-fixed paraffin sections were also stained with the copper phthalocyanin method (CPM) of Klüver and Barrera (Pearse, 1960).

RNA was localized in paraffin sections of brains and thoracic ganglia which had been fixed in Lillie's AAF for one hour, rapidly dehydrated in 95% and absolute ethyl alcohol, cleared in methyl benzoate, and vacuum-infiltrated. Constituents which stained with Einarson's gallocyanin-chromalum method for nucleic acids (pH 1.64; Pearse, 1960) after incubation for 1–2 hours in distilled water (37° C.), but which were not stained in adjacent sections incubated for the same period in 0.73% ribonuclease (recrystallized 5 times, without protease; Mann Laboratories), were considered RNA. These constituents are also present in neurons which have been fixed in alcoholic Bouin's, and they bind methylene blue at a pH of 2.5. Their strongly acidic nature is in accord with the hypothesis that they do, indeed, represent RNA.

To estimate the extent CAHP was dyeing the RNA component of neurons, paraffin sections of brains which had been fixed either in 80% ethyl alcohol or Helly's were stained after ribonuclease digestion. Sections which had been incubated in distilled water for identical periods were also stained with CAHP and served as controls.

The performic acid-alcian blue (PAAB) method of Adams and Sloper for disulfide groups (Pearse, 1960) was applied to paraffin sections of Helly-fixed material. Controls for the PAAB technique consisted of adjacent sections which

had been treated in the same manner as test sections, save for the omission of performic acid oxidation. The tetrazolium mixture of Barrnett and Seligman (1954) was used in the alkaline tetrazolium reaction (Pearse, 1960). This test was also applied to paraffin sections of Helly-fixed material.

Neurosecretory Material Produced by A-Cells

Of the many nerve cell bodies in the cockroach brain, only a relatively small number in the pars intercerebralis contains inclusions which stain both purple with AF and intensely blue-black with CAHP (Fig. 1A). Using the scheme of classification proposed by Nayar (1955), these would be called "A-cells." Due to the presence of stainable granules within the axoplasm, processes emanating from these cells can be traced to the first cardiac nerves, and thence to the corpora cardiaca (Scharrer, 1952; Arnold, 1960). Scharrer (1952) has demonstrated that this stainable material accumulates proximal to a transection of the first cardiac nerves, and is markedly depleted distally, suggesting that it is normally transported along the axons to the corpora cardiaca, which serve as neurohemal organs.

Different A-cells in the same individual are not invariably filled to the same extent with neurosecretory material (NSM). In the pars intercerebralis of some roaches, for example, A-cells which are packed with NSM were found adjacent to cells which contain only small, separate clusters of fine ($< 0.5\ \mu$ in diameter) neurosecretory granules. It is not improbable that this appearance is correlated with different phases of secretory activity.

Granules of NSM stain identically in the A-cells, in their processes within the brain and first cardiac nerve, and in the corpora cardiaca (Fig. 1B, C). A-cell NSM is poorly preserved by alcoholic fixatives. Unlike the situation in certain vertebrates (Sloper, 1955), loss of NSM is not appreciably prevented by post-fixing in 10% formalin after initial fixation in 80% ethyl alcohol prior to paraffin embedding. The loss of cockroach A-cell NSM appears to be due to the solvent action of alcohol, not to subsequent extraction by water. This is also indicated by the following data which show that NSM contains a lipid component.

Careful examination of adjacent Helly-fixed paraffin sections, one series stained with SBB, the other with CAHP, revealed that A-cell NSM is moderately sudanophilic. SBB can be removed readily with acetone, and the section can be restained, which further indicates that the sudanophilia is specific (Lillie, 1954). The sudanophilia of this NSM is also demonstrable in frozen sections of formal-bichromate or aqueous Bouin-fixed material. It is not apparent in frozen sections after hot chloroform:methanol extraction. A-cell NSM stains blue after Klüver and Barrera's CPM, and blue-black in frozen sections stained with Baker's AH (Fig. 1C). The latter reaction is abolished by hot pyridine extraction. The cytochemical evidence, therefore, suggests the presence of a phospholipid.

A-cell NSM is moderately PAS-positive in diastase-digested sections of Helly-fixed brain and retrocerebral complex. A positive reaction is also manifested by controls which have not been oxidized with periodic acid, but it is not as intense as that obtained after oxidation. After blocking exposed aldehyde groups with aniline acetate the PAS reaction is negative. The significance of the positive reaction is questionable. It could be caused by lipids (Pearse, 1960), or it could signify the presence of a carbohydrate moiety containing 1:2 glycol groups, which

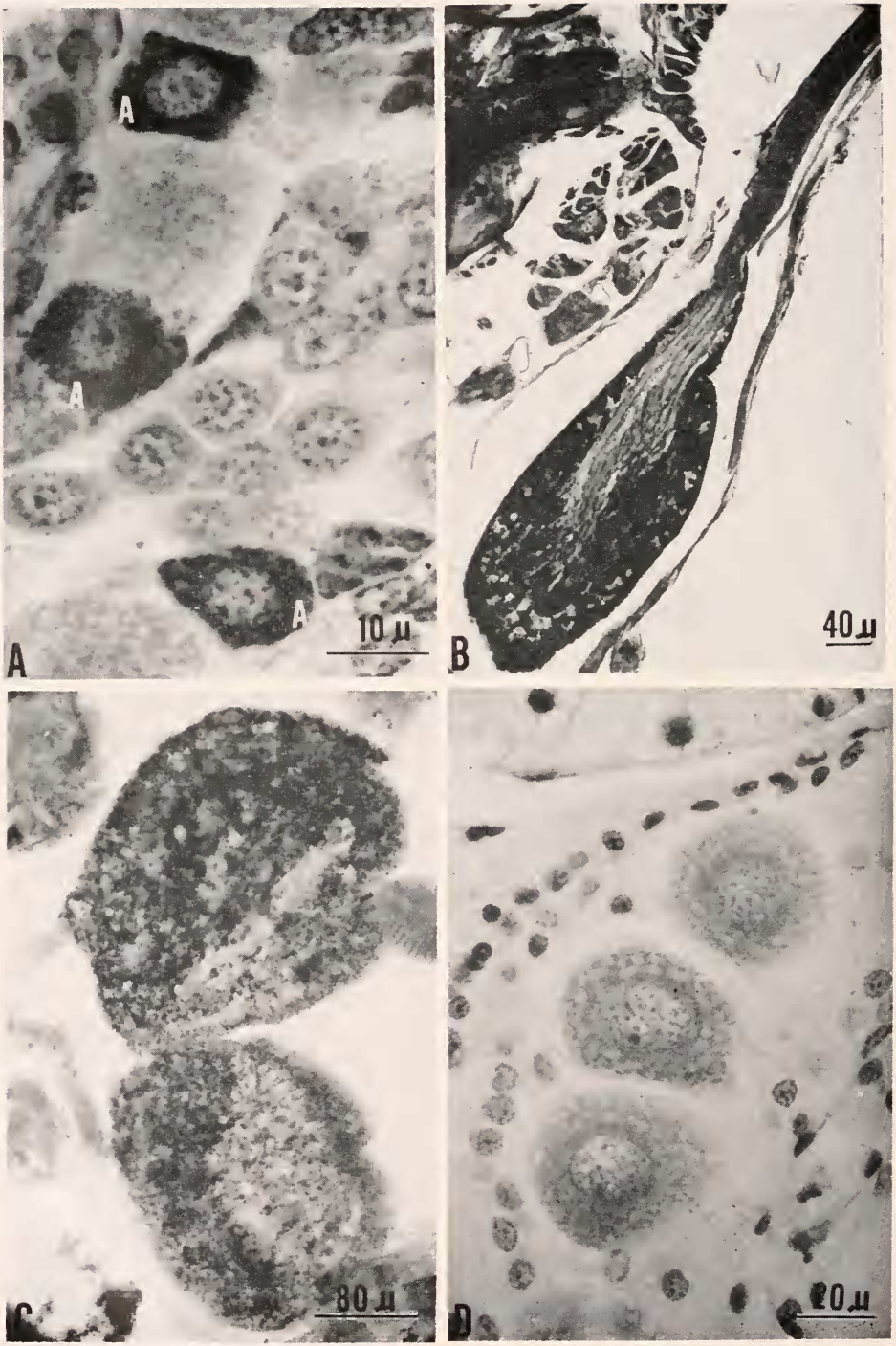

FIGURE 1.

occurs together with lipid. Since A-cell NSM is removed by lipid solvents, current cytochemical staining techniques do not permit a choice to be made between these possibilities.

A-cell NSM stains blue with the alkaline tetrazolium reaction, indicating the presence of protein reducing groups. Such an interpretation is further supported by the positive reaction noted with the PAAB method of Adams and Sloper for protein disulfide. The PAAB reaction is negative in controls which have not been oxidized with performic acid prior to staining with alcian blue. This is in agreement with the findings of Sloper (1957) in the cockroach *Leucophaea*. It should be emphasized, however, that this method stains other cytoplasmic inclusions in the nervous system besides A-cell NSM. Thus, cockroach gliosomes, first described by Scharrer (1939) and studied more recently by Pipa (1961), give a

TABLE I

Staining reactions of cytoplasmic inclusions in the brain of Periplaneta americana (L.)

Stain or test	A-cell NSM	Beta-granules	Delta-granules	RNA	Gliosomes
CAHP	Blue-black	Red	Pink or unstained	Blue-black	Blue-black
AF	Purple	Purple	Green	.....	Purple
Alkaline tetrazolium	Blue	Red	Gray	.....	Red
PAS (diastase)	++	++++	++++	.....	++++
PAS (unoxidized)	+	Faint, if at all	+	.....	+
PAS (blocked)	–	–	–	.....	–
SBB (frozen or Helly-fixed paraffin)	++	++++	++++	.....	Variable
SBB (lipid extracted)	–	–	–	.....	–
AH	++++	Faint, if at all	++++	.....	Variable
AH (hot pyridine extracted)	–	–	–	.....	–
Presence after alcohol fixation and paraffin method	Greatly reduced or absent	Greatly reduced or absent	Absent	Present	Greatly reduced or absent
CPM	++	++++	++++	.....	Variable
Gallocyanin-chromalum	–	–	–	++++	++++

positive reaction. Unlike neurosecretory granules, though, these inclusions stain red with the alkaline tetrazolium reaction. They also stain blue-black with Einarson's gallocyanin-chromalum, while A-cell NSM stains slightly, if at all (Table I).

RIBONUCLEIC ACID

A component cytochemically identifiable as RNA is present in the majority of neuronal perikarya. It is found distributed in patterns which vary in different

FIGURE 1. Photomicrographs of histological and cytological preparations; A, B, D fixed in Helly's; C fixed in 10% formalin followed by postchromation. (A) A-cells in pars intercerebralis stained with CAHP; (B) longitudinal section through corpus cardiacum and its nerve, stained with AF. Note intensely stained A-cell NSM in axons of nerve and in periphery of gland; (C) gelatin-imbedded frozen section of two corpora cardiaca; cross-section, stained with Baker's AH method for phospholipid; (D) three thoracic ganglion neurons stained with Einarson's gallocyanin-chromalum method for nucleic acids. A, A-cells.

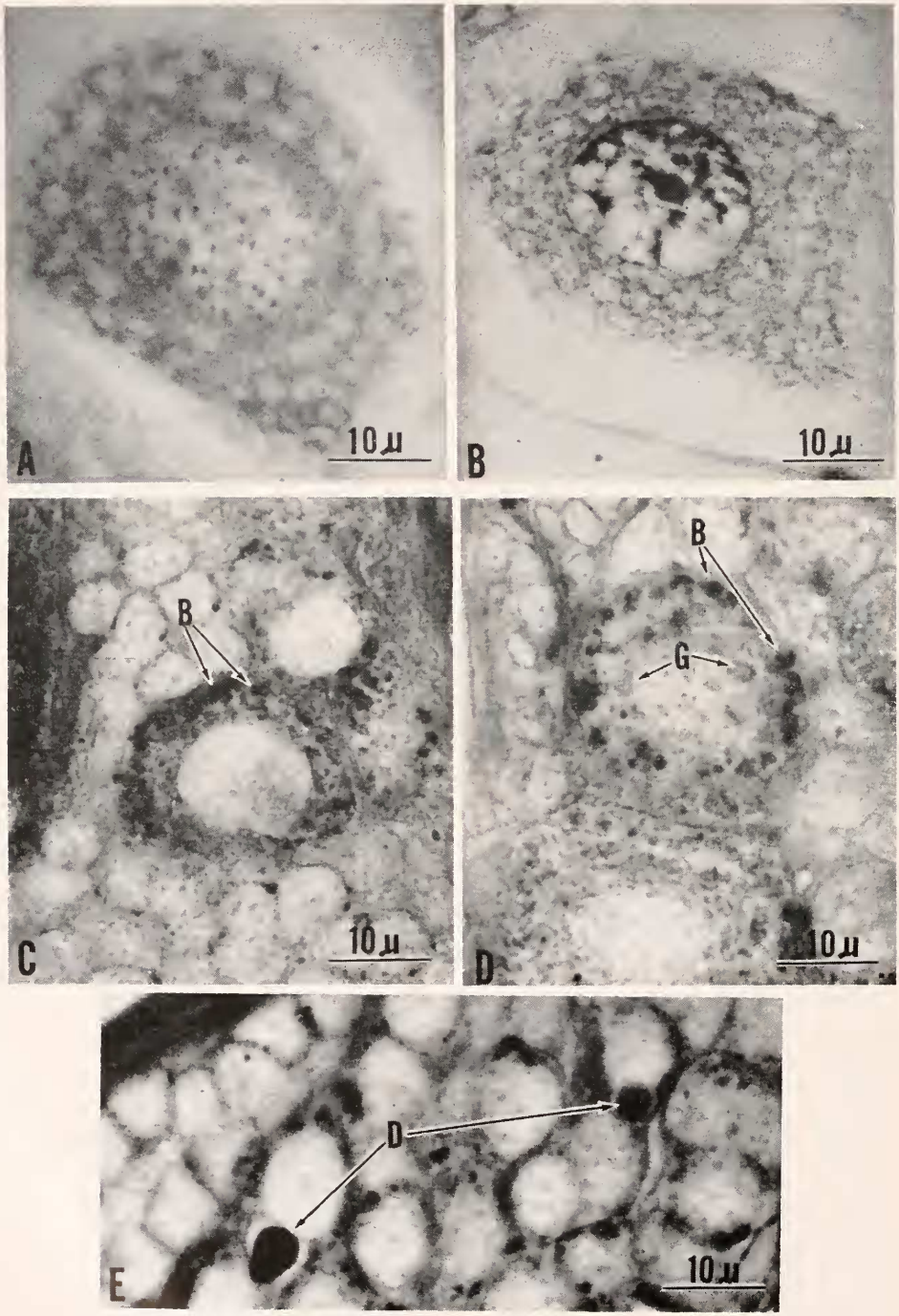

FIGURE 2.

neurons, commonly surrounding gallocyanin-chromalum-refractory spheroidal zones (Figs. 1D, 2A, B). Where these zones are reduced in diameter the distribution is more homogeneous. In agreement with the observations of Hess (1958), Arnold (1960), and Wigglesworth (1960), RNA was never found clumped to form well-defined Nissl bodies such as those described in vertebrate neurons. After alcohol fixation a vast majority of nerve cell perikarya in the brain stains blue-black with CAHP. That this staining is due to RNA is strongly suggested by the fact that it is abolished by ribonuclease digestion. Nerve cells of adjacent sections which were used as controls in this test stained after incubation in distilled water.

The perikarya of Helly-fixed neurons stain blue-black after CAHP, too. This is particularly noticeable when the phloxine counterstain is omitted. It is not surprising that this stainability remains essentially unaffected by ribonuclease, for this enzyme would hardly be expected to digest RNA in cells which have been fixed in a solution containing mercuric chloride. In all likelihood, though, the faint blue-black staining demonstrable in most Helly-fixed neurons after chrome alum-hematoxylin is also due to RNA. Fortunately, NSM within Helly-fixed A-cells stains more intensely than RNA after CAHP, and it is not difficult to distinguish these cells from other neurons found in the pars intercerebralis by this method.

Beta-Granules

A majority of the neurons contains inclusions which stain intensely with PAS, SBB, CPM, and AF, but which are not stained blue-black by CAHP (Fig. 2C, D). These inclusions range from 0.5 to 3.0 μ in diameter. They are especially well-preserved in frozen sections of formalin-bichromate-fixed material, and in Helly-fixed paraffin sections. They appear to vary in abundance in different neurons of the same brain, being most conspicuous in the axon hillock and periphery of each perikaryon, where they often occur in clusters. These inclusions were never found well-within the neuron process, and there was no evidence to support the contention that they represent neurosecretory products. Failure to find cytological evidence of axonal transport, however, does not eliminate the possibility that these inclusions represent a visible manifestation of products which leave the perikaryon via a different route, or in an invisible form. Although not all of these inclusions may be of the same type, they shall be designated beta-granules for the sake of discussion.

When counterstained sufficiently and not destained excessively, beta-granules demonstrate a slight phloxinophilia after CAHP, but this is less intense than that seen in nucleoli present in the same cell. They also stain red after the alkaline tetrazolium reaction, and are PAAB-negative.

The larger of these inclusions stained slightly, if at all, during the Baker AH procedure, whereas NSM of the A-cells, and inclusions believed to be mito-

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Figure 2. Photomicrographs of cytological preparations; A, C, D, E fixed in Helly's; B in Lillie's AAF. (A) thoracic ganglion neuron stained with Einarson's gallocyanin-chromalum method for nucleic acids. Note distribution of RNA around unstained spheroids which are believed to represent loci of Golgi apparatus; (B) similar to (A), but after alcoholic fixation; (C) Beta-granules stained with PAS and SBB; (D) Golgi apparatus and beta-granules in same neuron perikaryon, stained with PAS and SBB; (E) Delta-granules stained with PAS and SBB. B, beta-granules; D, delta-granules; G, Golgi apparatus.

chondria, were intensely stained in the same sections. Beta-granules stain with SBB and PAS in gelatin-imbedded frozen sections after formalin-bichromate or aqueous Bouin fixation. They are seldom found in frozen sections of brains which have been both fixed and extracted with hot chloroform:methanol. The larger of these inclusions are still abundant and stainable with SBB and PAS in frozen sections of material which has been fixed in aqueous Bouin's and subsequently extracted with hot chloroform:methanol, or hot pyridine. These data suggest that beta-granules contain a lipid or glycolipid which is either not predominantly phospholipid, or from which phospholipid has been removed during fixation. They are rendered relatively resistant to the action of lipid solvents by formalin fixation, indicating chemical association with a protein.

Although beta-granules stain alike, it would be gratuitous to assume that they are chemically identical. The cytochemical tests used are generally considered specific for certain classes of compounds if appropriate controls are employed, but they do not identify compounds within each class. The presence of a common chemical group within two inclusions can hardly be considered proof that the two are chemically identical.

It might be argued that certain, if not all of these "beta-granules" represent mitochondria. Though this has not been dismissed as a possibility, it seems unlikely in view of the fact that inclusions believed to be mitochondria on the basis of electron microscope data generally stained yellowish green rather than purple with AF. Mitochondria also stain less intensely with PAS than do beta-granules. After Baker's AH test mitochondria stain blue-black, whereas the larger beta-granules within the same cells stain slightly, if at all. Preliminary electron microscope data which are currently at hand likewise indicate that beta-granules differ in fine structure from "typical" mitochondria found within the neuron.

Golgi Complex

When formalin-bichromate-fixed frozen sections, or Helly-fixed paraffin sections of neurons are stained with SBB, not only are the homogeneously-stained beta-granules mentioned above made evident, but sudanophilic crescent-shaped bodies and "hollow" spheroids frequently appear in the same cells. Each of these is comprised of an "externum" which stains intensely with SBB, and an "internum" which is variably sudanophilic (Figs. 2D, 3A). The "externa" are especially well-revealed in paraffin sections stained with Klüver and Barrera's CPM (Fig. 3B).

When Helly-fixed paraffin sections that have been stained with PAS and counterstained with SBB are flooded with acetone, SBB is removed, and the following observations can be made during the destaining process: (1) The beta-granules described above are both intensely PAS-positive and sudanophilic. Thus, when SBB has been removed they are still clearly evident. (2) The "hollow" spheroids and crescent-shaped structures which stained with SBB become indiscernible. Unlike beta-granules these structures stain slightly if at all with PAS. (3) Occasional beta-granules occur within the "hollow" spheroids or crescents. When SBB has been removed, red-staining inclusions of variable size can be seen within a zone which is either PAS-negative, or only very slightly PAS-positive.

The Golgi apparatus in the neurons, then, consists of sudanophilic, crescent-

shaped or spheroidal "externa" which are probably manifestations of lamellar membrane systems (Beams *et al.*, 1953; Palay and Palade, 1955; Bern *et al.*, 1961). These are frequently closely associated with variably sudanophilic "interna" which may contain beta-granules. The beta-granules are sudanophilic, intensely PAS-positive, and stain purple with aldehyde fuchsin. A summary of their staining characteristics is given in Table 1.

Nerve cells which have been stained by techniques used to demonstrate RNA (*i.e.*, gallocyanin, cresyl violet) commonly present a variably "mottled" appearance (Figs. 1D, 2A, B). As previously noted, this is due to the staining of RNA

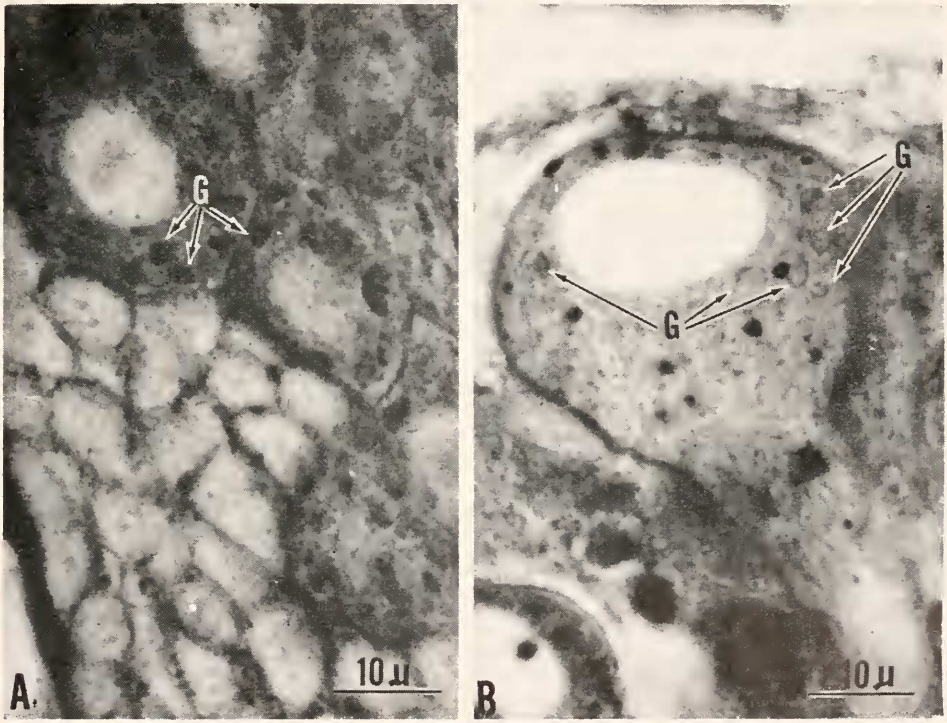

FIGURE 3. Photomicrographs of cytological preparations; fixed in Helly's. (A) Golgi apparatus stained with SBB. (B) Neuron stained with CPM; Golgi "externa" evident in cytoplasm. G, Golgi bodies.

which surrounds unstained spheroidal zones. An examination of adjacent Helly-fixed paraffin sections of the same neurons, one series stained with chrome alum-hematoxylin, the other with SBB, led to the conclusion that these spheroidal zones represent the loci of the Golgi apparatus which are devoid of ribosomes (*i.e.*, are present as "agranular reticula"; Palay and Palade, 1955; Palay, 1958; Bern *et al.*, 1961).

When frozen sections of aqueous Bouin-fixed, pyridine-extracted brains are stained with SBB and examined, the Golgi apparatus is no longer demonstrable. The cytoplasm now appears finely granular, save for the occurrence of the larger

beta-granules which continue to stain with SBB and which will also stain intensely with PAS. This may be taken as additional evidence that the Golgi apparatus and larger beta-granules are not one and the same structure.

Delta-Granules

Within the perikarya of certain of the smaller neurons (nuclear diameters of 10 to 15 μ) single, conspicuous, spherical inclusions are consistently encountered (Fig. 2E). These inclusions, here termed delta-granules, range from 2 to 5 μ in diameter. They occur within the brains of late instar nymphs, as well as in adult males and females. It is uncertain whether they are limited in occurrence to special cells, or if they occur sporadically in different neurons. Cells containing delta-granules may occur singly or in small groups. They were seen most frequently in the pars lateralis, less commonly in the pars intercerebralis.

Delta-granules do not stain homogeneously, for areas which fail to stain, or which stain unlike the remainder are noticeable at their centers. Delta-granules may stain faintly pink after CAHP, or they may fail to stain altogether. They are not fuchsinophil, but stain green with the counterstain employed in the AF technique. Their failure to stain purple, and the fact that they stain blue-black after Baker's AH distinguishes them from beta-granules (Table I). Since delta-granules no longer stain with AH after pyridine extraction it is likely that they contain appreciable phospholipid.

Discussion

The inherent limitations of staining reactions as criteria for categorizing cytoplasmic inclusions are painfully apparent. The fact that two structures stain alike does not necessarily mean they are chemically identical. When more rigorous cytochemical staining techniques are employed one may be led to conclude that two inclusions contain the same class of compounds, but it is seldom possible to tell precisely what the compounds are. It is common knowledge that structures which play disparate roles in the economy of the cell may contain identical classes of compounds, and may therefore stain identically. When two inclusions fail to stain alike, however, it is logical to consider them chemically or physically dissimilar.

In light of these precepts the advisability of using a diversity of staining techniques becomes obvious. In the present case such an approach has led to the recognition of three types of neuronal inclusions which are believed to exist in addition to mitochondria, ribosomes, and the agranular reticulum (*i.e.,* Golgi apparatus). To facilitate discussion these are called A-cell NSM, beta-granules, and delta-granules.

A-cell NSM was found in certain neurons of the pars intercerebralis, as well as in the corpora cardiaca. This is in agreement with the observations of Scharrer (1952) and Arnold (1960). The histochemical evidence indicates that this NSM contains a lipoprotein. This has also been noted in certain Lepidoptera (Rehm, 1955) and Hemiptera (Nayar, 1955). The fact that A-cell NSM stains intensely with Baker's AH before pyridine extraction, but not after, suggests the presence of phospholipid. The moderately PAS-positive reaction which this NSM exhibits is of unknown significance. That it is not due to glycogen is certain, but it

could be caused by a lipid component, or by a carbohydrate moiety associated with lipid. Since lipid solvents will remove A-cell NSM, current cytochemical staining procedures do not permit a choice to be made from among these possibilities.

As indicated by Sloper (1957) NSM produced by insect A-cells resembles that produced by certain neurons of the vertebrate hypothalamus, inasmuch as it shows evidence of containing a protein or polypeptide rich in cystine or cysteine. In other respects it differs significantly from vertebrate NSM, as reported by Sloper (1955), and Howe and Pearse (1956). These workers concluded that vertebrate NSM is mainly protein or polypeptide in nature, there being no indication of lipid. Their findings are in opposition to those of Schiebler (1952) who interpreted his histochemical data as indicating a glycolipoprotein complex.

Beta-granules are of variable size. Although they stain alike, it is possible that future study may reveal this to be a heterogeneous category. They occur in variable abundance in the majority of neuron perikarya, and are most conspicuous in the periphery, especially in the axon hillock region. A resumé of their staining characteristics is given in Table I. Beta-granules differ from mitochondria by staining intensely PAS-positive, and by staining purple with AF. More significantly, preliminary electron microscope data indicate that they differ from mitochondria in fine structure. Beta-granules were never found well-within the neuron process. If they do represent a type of neurosecretory material, they must be altered in size or stainability before passing out of the perikarya. Perhaps they are comparable to the chromophilic intraneuronal granules described by Thomas (1951).

Although it is not the intention of this study to enter into the "Golgi controversy" (Baker, 1957; Lacy and Challice, 1957; Palay, 1958), a comparison between locust "lipochondria" and structures noted here would seem appropriate. According to the interpretation of Shafiq (1953, 1954) and Shafiq and Casselman (1954) the classically described Golgi apparatus is an artifact resulting from the deposition of osmium or silver on the surfaces of homogeneous spheroids or "lipochondria" during impregnation. Malhotra (1956), however, reported seeing the binary spheroidal Golgi apparatus not only in osmium or silver impregnations, but also in living insect neurons, as well as in cytological preparations stained with SBB. He did not describe neuronal perikaryal inclusions other than mitochondria, Golgi binary spheroids (=lipochondria), and RNA particles.

The present account concurs with Malhotra's finding that in cytological preparations stained with SBB the Golgi apparatus is demonstrable as intensely stained crescentic "externa" associated with less intensely stained "interna." This appearance, then, is not entirely an artifact due to the deposition of osmium or silver on otherwise homogeneous spheroids. Electron microscope investigations have also revealed variably crescentic lamellar membrane systems ("agranular reticula") as part of the neuron Golgi apparatus (Beams *et al.*, 1953; Palay and Palade, 1955; Palay, 1958; Bern *et al.*, 1961), thus providing a probable fine-structure basis for the Golgi "externum" visible with the light microscope. The fact that no RNA could be demonstrated within the spheroidal zones ("externum" plus "internum") in the present work supports the contention that they may, indeed, represent the loci of the agranular (ribosome-free) reticula.

It is not clear whether by the term "lipochondria" Shafiq meant the spheroidal

zones (Figs. 1D, 2A, B) which represent the loci of the Golgi apparatus ("externum" plus "internum"), the larger beta-granules noted here, or both. It is quite certain, however, that the Golgi apparatus and the beta-granules are not one and the same structure. This interpretation is also supported by preliminary electron microscope data currently at hand.

The functional significance of the conspicuous delta-granules is likewise unknown. They were found solely in the perikarya of smaller neurons; never in the processes. A comparison between the staining affinities of these and certain other inclusions within the neurons is made in Table I.

The cytomorphology of neurons varies in different cells of the same brain. It also varies in the same group of cells in different brains. This variability is expressed as differences in the degree of Golgi apparatus elaboration, RNA distribution, and in number and size of inclusion granules. If we are willing to accept these static appearances as evidence for secretory activity, then it would seem that a majority of the neurons in the brain are secreting. Such cytomorphological features need not necessarily be correlated with neurosecretion, however, for they could conceivably reflect axoplasm replacement (Weiss and Hiscoe, 1948), or changes associated with ageing (*i.e.*, accumulation of lipofuscin).

On the basis of the cytological data presented here it would be premature to conclude that beta- and delta-granules represent neurosecretory products. It is also entirely possible that secretion may occur without the production of visible granules. Clearly, reciprocal physiological information is essential before this matter can be decided.

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Summary

1. A variety of cytological and cytochemical staining reactions has been applied in a study of neuronal inclusions in the brain of the American cockroach. Three types of cytoplasmic inclusions, which exist in addition to mitochondria, ribosomes, and the Golgi apparatus, are characterized. For discussion purposes these are called A-cell NSM, beta-, and delta-granules.

2. The cytochemical staining reactions used indicate that A-cell NSM is a cystine- or cysteine-rich phospholipoprotein.

3. Perikaryal inclusions which stain intensely with PAS, SBB, CPM, and AF, but which are not stained blue-black with CAHP, occur in the majority of neurons. These are called beta-granules. Despite similarities in stainability, there is a possibility that these inclusions are not all of one type. They clearly differ from the Golgi apparatus, and it is also highly unlikely that they are "typical" mitochondria. The cytochemical data suggest that beta-granules contain a lipid or glycolipid which is either not predominantly phospholipid, or from which phospholipid has been removed during fixation. The fact that they are rendered relatively

resistant to lipid solvents by formalin fixation indicates chemical association with a protein.

4. Delta-granules occur as single, conspicuous inclusions within the perikarya of certain of the smaller neurons. Unlike beta-granules, they stain green with AF. They appear to consist of a lipoprotein or glycolipoprotein which contains phospholipid.

5. The Golgi apparatus consists of sudanophilic crescent-shaped or spheroidal "externa" frequently closely associated with variably sudanophilic "interna" which may contain beta-granules. The "mottled" appearance of nerve cells which have been stained to demonstrate RNA appears to be due to the unstained Golgi spheroids.

6. The limitations of staining reactions as criteria for categorizing cytoplasmic inclusions, and the significance of cytomorphological features as indices of neurosecretory activity are discussed.

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